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Lipoxin A_4 Activates Alveolar Epithelial Sodium Channel Gamma via MicroRNA-21/PTEN/AKT Pathway in Lipopolysaccharide-Induced Inflammatory Lung Injury

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Abstract

Lipoxin A_4 (LXA_4) as endogenously produced lipid mediators promotes the resolution of inflammation. Previously, we have demonstrated that LXA_4 stimulated alveolar fluid clearance (AFC) through alveolar epithelial sodium channel gamma (ENaC-γ). In this study, we sought to investigate the mechanisms of LXA_4 in modulating ENaC-γ on LPS-induced inflammatory lung injury. MiR-21 was up-regulated in LPS challenging and down-regulated by LXA_4 administration *in vivo* and *in vitro*. Serum miR-21 concentration was also elevated in acute respiratory distress syndrome (ARDS) patients as compared with healthy volunteers. LPS increased miR-21 expression by activation of activator protein 1 (AP-1). In A549 cells, miR-21 up-regulated phosphorylation of AKT activation via inhibition of PTEN therefore reduced the expression of ENaC-γ. In contrast, LXA_4 reversed LPS-depressed ENaC-γ expression through inhibiting AP-1 and activating PTEN. In addition, miR-21 inhibitor mimicked the effects of LXA_4; over-expression of miR-21 abolished the protective effects of LXA_4. Finally, both AKT and ERK inhibitor (LY294002 and UO126) blocked LPS's effects on the depression of ENaC-γ. However, LXA_4 only inhibited LPS-induced phosphorylation of AKT. In summary, LXA_4 activates ENaC-γ in part via miR-21/PTEN/AKT signaling pathway.

Abbreviations

AFC: alveolar fluid clearance

AP-1: activator protein 1

ARDS: acute respiratory distress syndrome

CDS: coding sequence

ENaC: epithelial sodium channel

LPS: lipopolysaccharide

LXA_4: lipoxin A_4

PTEN: phosphatase and tensin homolog

Acute respiratory distress syndrome (ARDS) is a devastating syndrome characterized by dysregulated inflammation and alveolar barrier disruption that impairs pulmonary gas exchange, leading to refractory arterial hypoxemia and respiratory failure [1, 2]. Bacterial or viral pneumonia and sepsis are the most common causes of acute lung injury (ALI) and ARDS [1], wherein Gram-negative bacteria are a prominent cause. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, is one of the mainly pro-inflammatory reaction factors in ALI, leading to neutrophil recruitment and pulmonary edema[3]. After decades of efforts, ARDS has a declined mortality, perhaps secondary to more widespread use of lung-protective ventilation, reductions in nosocomial infections and supportive care[2, 4].

Recently, the critical importance of timely and effective removal of excessive alveolar edema, namely alveolar fluid clearance (AFC), has being better recognized in ARDS[5, 6]. It is now well established that the mechanisms of AFC is the alveolar fluid cleared by active Na^+ transport [7], which across the alveolar epithelium via apical alveolar epithelial sodium channel (ENaC) [8-10] and through basolateral Na^+-K^+-ATPases [9, 11], then the water passively moves from the air spaces to the alveolar intersititium as the result of transepithelial osmotic gradient caused by active Na^+ transport. Hence, both ENaC and Na^+-K^+-ATPases are pivotal in timely and effective removal of excessive alveolar edema fluid [12].

There is a growing appreciation that there have short-term regulatory and long-term regulatory mechanisms in active Na^+ transport. Short-term regulatory mechanisms mainly possessed by dopamine and β-adrenergic agonists; transcriptional and posttranscriptional mechanisms response to long-term regulation [11]. We previously reported that intravenous β-agonists (salbutamol) reduced extravascular lung water in ARDS patients[13-16]. However, we found salbutamol significantly increased 28-day mortality because of its side effects such as tachycardia, arrhythmias, and lactic acidosis in a multicenter, randomized, controlled clinical trial[17]. Therefore, we need to identify some new therapeutic agents.

Lipoxins (LX) are eicosanoids formed during inflammation via transcellular biosynthetic routes that posses distinct anti-inflammatory and proresolution properties, including the repression of proinflammatory cytokine production, inhibition of leukocyte-mediated injury, and stimulation of macrophage clearance of apoptotic neutrophils [18, 19]. LXA_4 have emerged as founding members of the first class of lipid mediators that can function as “braking signals” in inflammation, and are “switched on” in the resolution phase of an inflammatory response. We previously described distinct pro-resolution and anti-edema properties of LXA_4 and aspirin-triggered LXA_4 in rat ALI model[10, 20, 21]. We found LXA_4 significantly stimulated AFC through up-regulated ENaC-γ protein expression[10], which presents as regulative subunit of ENaC [22]. Thus, LXA_4 is a useful tool and offer leads for developing novel therapeutic intervention.

MicroRNAs (miRNAs) are a class of gene products that recently were implicated in several lung diseases[23-25]. Importantly, miRNAs emerge as novel biomarkers [26] and therapeutic strategies[27]. Recently studies revealed that miR-21 was dynamically regulated in LPS-induced ALI[28, 29]. Also, researches have showed that miR-16 up-regulated ENaC-β [30], miR-101 and miR-144 targeted cystic brosis transmembrane conductance regulator (CFTR) 3’ UTR [31], miR-96 and miR-330 bound to the 3’ UTR of aquaporin 5 (AQP5) [32]. Previous studies demonstrated that each specialized pro-resolving mediators (SPM) regulated a distinct panel of miRNAs[33-35]. More recently, LXA_4 exhibited antifibrotic properties by up-regulation let-7c expression in renal fibrosis[36]. However, whether LXA_4 augment AFC through a particular miRNA in LPS-induced ALI, if so, what the underlying mechanisms are, remain unclear.

In the present study, we investigated the central role on miR-21 in LPS-dependent inflammatory lung injury. Based on *in silico* bioinformatic analysis and real-time PCR, we focused on miR-21 and its potential target ENaC-γ mRNA. We also evaluated ARDS patients serum miR-21 level. Additionally, we investigated

the effect of LXA_4 on the protein expression of ENaC-γ and PTEN, and the phosphorylation of AKT and ERK *in vitro*. Finally, to better understand the mechanisms of action of LXA_4, we used miR-21 mimic, miR-21 inhibitor, AP-1 inhibitor (SR11302), AKT inhibitor (LY294002), and ERK inhibitor (UO126) to investigate how this signaling pathway regulates ENaC-γ protein expression.

Materials and Methods

Reagents

LPS, LY294002 (AKT inhibitor) and U0126 (ERK inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO). LXA_4 was from Cayman Chemical Company (Ann Arbor, MI). SR11302 (AP-1 inhibitor) was purchased from Tocris Bioscience (Bristol, UK). RPMI 1640, fetal bovine serum (FCS), trypsin and enzyme-free cell dissociation buffer were purchased from Gibco (Grand Island, NY). Penicillin and streptomycin in saline citrate buffer were from Invitrogen (Carlsbad, CA).

Animals

Specific pathogen-free adult male SD rats, weighing 250-300g, obtained from Slac Laboratory Animal (Shanghai, China), were housed under controlled temperature and humidity in a day-night cycle, with free access to standard laboratory food and water. The study was approved by Animal Studies Ethics Committee of Wenzhou Medical University.

Cell culture

A549 Cells were cultured in RPMI 1640 containing 10% FCS with 100 U/ml penicillin, and 100 μg/ml

streptomycin at 1 x $10^6/cm^2$, and incubated in a humidified atmosphere with 5% CO_2 at 37°C. Before stimulation or transfection, cells were cultured overnight in 1% FBS.

Study participants and sample processing

All patients and healthy volunteers provided written informed consents and this study complied with the declaration of Helsinki and was approved by the Committee on Ethics of the Second Affiliated Hospital of Wenzhou Medical University. Study enrollment occurred between January to June 2014. ARDS was defined and classified according to the Berlin definition [37]. Following the Berlin definition, diagnostic criteria for ARDS rely on 4 categories: 1) timing: within 1 week of a known clinical insult or new or worsening respiratory symptoms; 2) radiography: bilateral opacities - not fully explained by effusions, lobar/lung collapse or nodule; 3) origin of lung edema: respiratory failure not fully explained by cardiac failure or fluid overload, and 4) oxygenation impairment: subdivided into 3 categories according to the degree of hypoxemia severity (mild, moderate and severe). Eligible patients were at least 18 years of age and diagnosed within 48 hours with a PaO_2/FiO_2 ratio of < 300. Exclusion criteria included pre-existing severe disease of any major organs, pregnancy, pulmonary hypertension, malignant disease, human immunodeficiency virus (HIV) infection or if informed consent could not be obtained. Volunteers were not taking any medications for 2 weeks before commencement of the study.

Blood from ARDS patients and healthy volunteers were put into EDTA-containing tubes (BD Biosciences, New Jersey, USA). Tubes were rotated end-over-end at room temperature for 30 min and centrifuged (795 × g, 20 min, 4 °C) by Beckman JB-6 (Beckman Coulter, Danvers, MA). The serum samples were aliquoted into 1.5-mL RNase-free Eppendorf tubes (Ambion, Carlsbad, CA).

Bioinformatics analysis and miRNA prediction

The microRNA databases and target prediction tool TargetScan 6.2 (Cambridge, MA) (http://www.targetscan.org/index.html) [38] and miRWalk (Mannheim, Germany) (http://mirwalk.uni-hd.de/) [39] were used to identify potential microRNA targets.

Transfections and reporter assays

Cells were transfected with miR-21 mimic, inhibitor, or negative controls (RiboBio, Guangzhou, China) according to manufacturer's protocol.

Human SCNN1G (ENaC-γ mRNA) coding sequence (CDS) (828-835 bp) containing the putative binding sites of miR-21 were amplified by PCR, inserted into the firefly luciferase reporter vector pmiR-RB-REPORT™ (RiboBio, Guangzhou, China) between the restrictive sites Xho I and Not I, and validated by sequencing. Their mutant constructs with a mutation of the miR-21 seed sequence were generated with the mutagenic oligonucleotide primers, according to the manual of GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). Cells were plated at $1*10^5$ cells/well on 24-well plates, and transfected with pmiR-RB-SCNN1G CDS (30 ng) or their mutant constructs. Twenty-four hrs post-transfection, firefly and Renilla luciferase activities were consecutively measured, according to the dual-luciferase assay manual (Promega, Madison, WI). The Renilla luciferase signal was normalized to the firefly luciferase signal for each individual analysis.

Reverse transcription and real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Pri-miR-21, ENaC-γ and PTEN mRNA were quantified by real-time PCR assay

using the SYBR Green real-time PCR Master Mix regents (Toyobo, Osaka, Japan) with GAPDH as the normalisation control. For determination of miRNAs expression, total RNA (2 μg) was reversely transcribed with Bulge-Loop miRNA-specific reverse transcription-primers (RiboBio, Guangzhou, China). Real-time PCR reactions were done with SYBR Green real-time PCR Master Mix regents (Toyobo, Osaka, Japan) and Bulge-Loop primers (RiboBio, Guangzhou, China) on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with small nuclear RNA U6 as the normalisation control. For determination of serum miRNA concentration, specific TaqMan assays for miRs and the TaqMan Micro-RNA Reverse Transcription Kit were used, followed by real-time PCR using the Universal PCR Master Mix (Ambion, Carlsbad, CA) according to the manufacturer's protocol with cel-miR-39 as the normalisation control.

Western blot

Western blotting analysis from A549 cells lysis solution was performed as described previously[10]. The protein extracts were separated in SDS-polyacrylamide gels and transferred to PVDF membranes. The primary antibodies used included the following: β-actin (1:2000; Santa, Santa Cruz, CA), GAPDH (1:5000; Bioworld, St. Louis, MN), ENaC-γ (1:1,000; Abcam, Cambridge, MA), PTEN (1:500; Abcam, Cambridge, MA), phospho-AKT (1:1000; Cell Signaling Technology, Boston, USA), AKT (1:1000; Cell Signaling Technology, Boston, USA), phospho-ERK (1:1000; Cell Signaling Technology, Boston, USA), ERK (1:1000; Cell Signaling Technology, Boston, USA). And after primary antibodies incubated overnight at 4°C, the secondary horseradish peroxidase-conjugated IgG which were either goat anti-mouse or goat anti-rabbit, were used at 1:3,000 dilution and imaged with the Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

221

222 **Statistical analysis**

223 Data are presented as means ± SEM. All data were analyzed by the Student's t test or by one-way ANOVA

224 followed by Tukey's post hoc test for multiple comparisons. Statistical analysis and graphs were done

225 with GraphPad Prism 5.0 (GraphPad, San Diego, CA). Results with $P < 0.05$ were considered statistically

226 significant.

227

228

229 **Results**

230 **Specific miRNAs are up-regulated in LPS challenging and down-regulated with LXA_4**

231 **administration**

232 *In silico* bioinformatic analysis (TargetScan and miRWalk) revealed that miR-181a/b, miR-340, miR-137

233 were likely targeting the Na,K-ATPase β1 3'-UTR, miR-29a/b/c were likely targeting the Na,K-ATPase α1

234 5'-UTR, and miR-21 was able to potentially regulate the expression of ENaC-γ mRNA by binding to its

235 CDS (Figure 1A). To investigate whether these miRNAs had roles in Na,K-ATPase α1, Na,K-ATPase β1 and

236 ENaC-γ mRNA post-transcriptional regulations, we next sought to determine whether LPS regulates

237 these miRNAs expression and the effects of LXA_4. Hierarchical clustering grouped these miRNAs into

238 distinct clusters based on their relative abundance at the different treatment, *in vivo* and *in vitro* (Figure

239 1B and C). In particular, among the few miRNAs highly expressed in the present of LPS, miR-21, miR-29b

240 and miR-340 displayed >2.5-fold increases compared with control group and attenuated in the LXA_4

241 treatment. Conversely, a large group of miRNAs showed less vigorous increase (<0.4-fold changes).

242 Among these, we focused on miR-21 because it showed consistent up-regulation at LPS stimulation and

down-regulation under LXA_4 treatment *in vivo* and *in vitro*. Therefore, we next determined the expression levels of miR-21 along we previously described LPS-induced rat ALI model [40] using real-time PCR. MiR-21 remained up-regulated at LPS present and down-regulated at LXA_4 treatment (Figure 2A). Consistent with results from *in vivo*, miR-21 was significantly up-regulated at the stimulation of LPS compared to the control group and attenuated in the LXA_4 treatment in A549 cells (Figure 2B).

MiR-21 serum concentration is elevated in ARDS

As a demonstration of the potential importance of miR-21, we investigated miR-21 serum concentration in ARDS patients (see Supplemental Table S1). While miR-21 serum concentration was elevated in ARDS patients (at admission to the ICU and prior to therapeutic interventions) compared to healthy volunteers (n=4) (Figure 2C).

AP-1 mediated LPS-induced miR-21 expression

In order to clarify the regulatory mechanism by which LPS and/or LXA_4 affect miR-21 expression, we analyzed primary transcripts of miR-21 gene (pri-miR-21) in A549 cells. LPS increased the amounts of pri-miR-21, whereas LXA_4 abolished the LPS-induced pri-miR-21 expression (Figure 3A), which consistent with the mature miR-21, indicating that LPS and LXA_4 regulate miR-21 biosynthesis at the transcriptional level.

For AP-1 is one of the conserved enhancer elements in the miR-21 promoter region[41, 42], we next explored whether AP-1 was involved in LPS and/or LXA_4 mediated miR-21 biosynthesis. Indeed, pretreatment of the A549 cells with the selective AP-1 inhibitor retinoid SR11302 [43] reduced the production of LPS-induced miR-21, in a dose- and temporal-dependent pattern. In all responses, the miR-21 expression was decreased dose-dependently with a concentration of 10nM producing a maximal effect

(Figure 3B). The dynamic expression of miR-21 decreased initially at 4 h with recovery occurring at 24 h and 6-12 h producing a maximal effect (Figure 3C). In subsequent experiment, the miR-21 expression was decreased 35% in the treatment of 10nM SR11302 for 12h, meanwhile, LXA_4 significantly decreased LPS-induced miR-21 expression (Figure 3D).

LXA_4 attenuates LPS-depressed ENaC-γ expression through inhibition of miR-21, but miR-21 does not target ENaC-γ mRNA directly

We previously demonstrated that LPS depressed ENaC-γ protein expression, and LXA_4 reversed the repression in primary rat alveolar type □ epithelial cells[10]. Therefore, we hypothesized that LXA_4 attenuated expression of LPS-induced miR-21 might underlie the protective mechanism of LXA_4. Firstly, we observed that LPS stimulated a significant reduction in ENaC-γ mRNA and protein expression. However, under the treatment of LXA_4 and SR11302, ENaC-γ expression was increased (Figure 4A and B). We therefore proposed that miR-21 maybe a pivotal mediator of LPS-deduced ENaC-γ expression. Secondly, to test this hypothesis, we transfected miR-21 mimic or inhibitor in A549 cells resulting in a 1000-fold induction or 2.2-fold repression in miR-21 expression levels, respectively (See Supplemental Figure S1). However, there was no significant change in ENaC-γ protein expression (Figure 4C and D). Thirdly, to further address weather miR-21 involved in LPS-depressed ENaC-γ expression, A549 cells stimulated with LPS (1 μg/ml) for 8 hours after transfection of miR-21 mimic aggravated LPS-depressed ENaC-γ protein expression and miR-21 inhibitor attenuated the depression (Figure 4E and F). These data suggested that up-regulation of miR-21 by LPS was necessary for subsequent down-regulation of ENaC-γ expression. Furthermore, miR-21 mimic abolished the protective effect of LXA_4 in attenuating LPS-mediated ENaC-γ depression (Figure 4G and H). Conversely, ENaC-γ protein expression was induced

upon miR-21 inhibitor transfection after LPS stimulation (Figure 4I and J). Together, these findings indicated that LXA_4 possessed protective effect upon ENaC-γ protein expression through down-regulated miR-21 expression. Finally, luciferase reporter assays containing wildtype and mutant seed region miR-21 binding site of the ENaC-γ mRNA CDS confirmed that there did not have the interaction between miR-21 mimic and this site (Figure 4K).

LXA_4 attenuated LPS deduced ENaC-γ expression via miR-21/PTEN pathway

Given the foregoing evidence for phosphatase and tensin homolog (PTEN) is down-regulated in LPS-induced rat ALI model[44], and miR-21 is a direct target of PTEN[45], we investigated miR-21 target gene PTEN expression in LPS challenging *in vitro*. A549 cells stimulated with LPS resulted in significant down-regulation of PTEN mRNA and protein. Conversely, LXA_4 alleviated this effect, meanwhile, SR11302 showed analogous result (Figure 5A, B and C). Moreover, transfection with miR-21 inhibitor significantly increased PTEN protein expression (Figure 5D and E). Furthermore, in A549 cells transfected with miR-21 inhibitor, miR-21 silencing prevented LPS-mediated depression of PTEN and ENaC-γ protein expression (Figure 5F, G, H and I). Together, these results indicated that miR-21/PTEN was the key signaling pathway underling the LXA_4 attenuated LPS-depressed ENaC-γ protein expression.

LPS decreased ENaC-γ protein expression in a miR-21/PTEN AKT and ERK dependent fashion

AKT and ERK are regulated by miR-21/PTEN pathway[46]. Therefore, we addressed whether these signaling pathways were involved in the regulation of LPS on ENaC-γ protein expression. Firstly, we tested the phosphorylation of AKT and ERK in LPS stimulated A549 cells. We observed phosphorylation of AKT and ERK with 30 minutes LPS stimulation in A549 cells, resulting significant increased phosphorylation of AKT and ERK (Figure 6A, B, C and D). A549 cells pretreated with AP-1inhibitor (SR11302), AKT inhibitor

(LY294002) and ERK inhibitor (U0126) abrogated LPS-decreased ENaC-γ protein expression (Figure 6E and F).

LXA_4 augmented ENaC-γ protein expression through attenuating LPS-induced phosphorylation of AKT

In the addition of SR11302, LY294002 and LXA_4 abolished LPS-induced up-regulation of phosphorylation of AKT (Figure 6A and B). However, only the addition of U0126 reversed LPS-induced up-regulation of phosphorylation of ERK, SR11302 and LXA_4 failed (Figure 6C and D). Under pretreatment of LXA_4, ENaC-γ protein expression significantly elevated compared with LPS group (Figure 6E and F). Our data indicated that LXA_4 attenuated LPS-depressed ENaC-γ protein expression in part through miR-21/PTEN/AKT pathway.

Discussion

In the present study, we have identified a distinct role of LXA_4 on miR-21 expression in LPS-induced inflammatory lung injury. Our data showed that miR-21 was up-regulated in LPS challenging and down-regulated with LXA4 administration *in vivo* and *in vitro* and serum miR-21 concentration was elevated in ARDS patients. MiR-21 up-regulated phosphorylation of AKT activation via inhibition of PTEN therefore reduced the expression of ENaC-γ. However, LXA_4 reversed LPS-depressed ENaC-γ expression through inhibiting AP-1 and activating PTEN indicating that LXA_4 activated ENaC-γ via miR-21/PTEN/AKT signaling pathway (as summarized in Figure 7).

We have reported the protective effects and anti-edema properties of LXA_4 in oleic acid (OA) and LPS-induced rat ALI model[10, 20, 21]. To date, mechanisms associated with LXA_4 in the resolution of AFC

remain of wide interest. Results presented herein are the novel evidences of miRNA expression profiling with treatment of LXA_4 in LPS-induced inflammatory lung injury. Our results have confirmed that LPS induced a sustained expression of miR-21 *in vivo* and *in vitro*. However, LXA_4 showed inhibition in miR-21 expression. Sterghios *et al.*[29], using mouse aerosilised LPS model, reported that miR-21 was up-regulated and showed time dependent increases, coincidence with our *in vivo* result. Lee *et al.* reported that miR-21 was also up-regulated throughout the 24 h following OA challenge, in time-course analysis of miRNAs expression in rat OA-induced ALI[47]. These *in vitro* and *in vivo* results suggest the importance of miR-21 in ALI. Coincidently, we also found augmentation in miR-21 serum concentration in ARDS patients. Therefore, miR-21 may become a novel biomarker to predict severity and outcome of ARDS in the future.

Pri-miR-21 also elevated in LPS stimulating and attenuated by LXA_4 treatment, which implied transcriptional regulatory mechanism. AP-1, which binding site located in miR-21 promoter region, proved to be one of the conserved enhancer elements of miR-21[41]. Previous studies demonstrated that LXA_4 and aspirin-triggered LXA_4 reduced AP-1 activity in the present of LPS[48]. Here we used AP-1 inhibitor SR11302 abolished LPS-induced miR-21 expression in a dose- and temporal-dependent pattern. Moreover, LXA_4 mimicked the effect of SR11302, through inhibition of the LPS-mediated AP-1 pro-inflammation signal, which as a consequence may lead to depression of miR-21.

ENaC-γ presents as regulative subunit of ENaC promotes the resolution of AFC [9]. Our results on miRNAs expression patterns and *in silico* predictions led us to propose that miR-21 mediated the effects of LXA_4 in promotion ENaC-γ expression in inflammatory lung injury responses. This hypothesis was supported by our findings that under transfecting miR-21 mimic and inhibitor into A549 cells. Over-expression of miR-21 abolished the protective effects of LXA_4 in LPS-deduced ENaC-γ expression; however, miR-21

353 inhibitor mimicked the effects of LXA_4. Our data clearly demonstrate that miR-21 had no effect on ENaC-γ
354 expression in physiological conditions. However, trasfection with miR-21 mimic or inhibitor exacerbated
355 or derepressed LPS-depressed ENaC-γ expression. Vaporidi K demonstrated that ectopic expression of
356 miR-21 reduced lung compliance and increased alveolar-arterial oxygen difference and protein levels in
357 bronchoalveolar lavage in ventilator-induced mouse ALI model[49]. Here, we observed that treated with
358 miR-21 mimic abolished the protective effect of LXA_4 on LPS-deduced ENaC-γ expression. Additionally,
359 both SR11302 and LXA_4 reversed LPS-decreased ENaC-γ protein expression. Our data demonstrates that
360 down-regulation of miR-21 is a necessary step in LXA_4 blunting LPS-deduced ENaC-γ expression.
361 Luciferase reporter assay demonstrated that miR-21 did not target ENaC-γ mRNA directly. However, we
362 observed the depression of PTEN expression under LPS stimulation, and prevented by LXA_4 and SR11302
363 pretreatment. Consistent with previous studies that up-regulated miR-21 depressed PTEN mRNA and
364 protein expression in ventilator-induced and OA-stimulated rat ALI model[47, 49]. In addition, PTEN contains
365 miR-21 binging site within 3' UTR[45]. Hence, we provide evidence linking LPS-mediated up-regulation of
366 miR-21 with down-regulation of PTEN mRNA and protein in alveolar epithelia. Furthermore, transfection
367 of miR-21 inhibitor increased PTEN protein expression. Consequently, loss of miR-21, LPS-depressed
368 PTEN and ENaC-γ were attenuated, demonstrating the importance of this miR-21/PTEN pathway to LPS
369 actions on PTEN and ENaC-γ expression. This observation unveils a novel pathway wherein miR-21/PTEN
370 responses for LXA_4 in augmenting ENaC-γ protein expression in LPS-stimulated inflammatory lung injury.
371 Both AKT and ERK are downstream regulators of miR-21/PTEN pathway[46]. In addition to this, it is known
372 that ENaC activity is regulated by a complex network of signaling pathways, including AKT and ERK
373 pathways[50]. We previously demonstrated that AKT pathway has been shown to mediate LPS-induced
374 decrease of CFTR protein expression in primary ATII cells[20]. LPS resulted in a rapid phosphorylation of

AKT and ERK, which reached the peak in 30 minutes[20]. In the current study, the addition of LY294002 abrogated LPS-induced phosphorylation of AKT, meanwhile, the pretreatment of SR11302 and LXA_4 decreased phosphorylation of AKT to average 30 min after LPS stimulation in A549 cells. Interestingly, pretreatment of SR11302 and LXA_4 failed to depress LPS-induced phosphorylation of ERK. It has been reported that Aspirin-triggered lipoxin A_4 inhibited myeloperoxidase (MPO) suppression of neutrophil apoptosis via down-regulation of AKT and ERK[51]. In contrast, Prieto P suggested that pretreatment with LXA_4 promoted a rapid activation of ERK after staurosporine challenge in RAW 264.7 cells[52]. These differences were probably a consequence of signaling convergence and cross talk between the AKT and ERK cascades.

Although multiple studies exist regarding the regulation of ion channels by miRNAs[30-32], our results demonstrate that miR-21/PTEN/AKT pathway involves in LXA_4 and LPS regulation of ENaC-γ protein expression. However, there are three aspects remain to be further addressed. Firstly, to verify the relationship between miR-21 and ARDS there needs to recruit more patients. Secondly, ENaC-γ activity and trafficking such as ion and fluid transport need to be additionally evaluated[53]. Finally, the mechanism we demonstrated based on our *in vitro* study; it would be of interest to further substantiate our findings that miR-21/PTEN/AKT pathway contributes to the lung anti-edema effects of LXA_4 *in vivo*.

In this study, we have shown that down-regulation of miR-21 regulated ENaC-γ expression via a PTEN/AKT signaling pathway after LXA_4 treatment in LPS-dependent inflammatory lung injury. However, its potential actions on other pathological mechanisms may be contributing as a crucial mediator. MiR-21 has been shown to affect ERK–MAP kinase signaling in cardiac fibroblasts, which in turn positively regulates cardiac fibroblast survival, leading to fibrosis, hypertrophy and cardiac dysfunction[54]. In a lately study demonstrated that miR-21 was activated and involved in the pathophysiologic processes of the

Ischemia/Reperfusion-induced acute kidney injury. Therefore, miR-21 can serve as potential targets for modulation by specific miR-21 antagomiRs[27] to achieve protection effects[55]. Furthermore, miR-21 has been demonstrated to be a multi-faced miRNA[42] and deregulated in almost all types of cancers and therefore was classified as an oncomiR[56], especially in cardiovascular and pulmonary diseases. Hence, pharmacologically down-regulation of miR-21, namely miR-21 antagomiR or agents like LXA_4, may be a new therapeutic entry point in the future.

In conclusion, these data demonstrate that LXA_4 alleviated LPS-depressed ENaC-γ protein expression via miR-21/PTEN/AKT signaling pathway. Thus, treatment with LXA_4 in down-regulation of miR-21 could represent novel targets in the treatment of the critically ill patients with inflammatory lung injury. Our findings reveal a novel mechanism of LXA_4 in reversing LPS-deduced ENaC-γ protein expression and LXA_4 may provide a new therapy for the resolution of inflammatory lung injury.

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Disclosures

The authors have no financial conflicts of interest.

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Titles and legends to figures

Figure 1: LXA_4-regulated miRNAs target genes with specific roles in alveolar fluid clearance. (A) The putative miRNAs binding sites locate in Na,K-ATPase β1, α1, or ENaC-γ mRNA. (B) SD rats were injected with LXA_4 (2 μg/kg) or vehicle (1 ml Saline) 8 hours after LPS (20 mg/kg) stimulation through caudal vein, lung tissue homogenate samples are harvested 1 hour later (n=3 per group), and miRNA fractions were isolated. (C) A549 cells treated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 hours (n=4). Heat map cluster represents relative expression of 8 miRNAs determined with real-time PCR after normalization with U6. Relative expression intensities are indicated in a green-red color code based on ΔC_t values.

Figure 2: MiR-21 is increased in inflammatory lung injury. (A) SYBR Green real-time PCR validation of miR-21 expression levels in rats ALI model. SD rats were injected with LXA_4 (2 μg/kg) or vehicle (1 ml Saline) 8 hours after LPS (20 mg/kg) stimulation through caudal vein, lung tissue homogenate samples are harvested 1 hour later (n=3 per group). (B) miR-21 expression measured by SYBR Green real-time PCR in A549 cells treated with LPS (1 ug/ml) and/or LXA_4 (100 nM) for 8 hours (n=4). (C) TaqMan real-time PCR detection of serum miR-21 level of ARDS patients at admission to ICU revealed (p=0.03, t-test) elevating of miR-21 in ARDS patients (n=4) as compared with healthy controls (n=4). Plots are displayed, where the long line indicates the median per group, and horizontal lines show 95% confidence interval. * $P < 0.05$ versus control group, # $P < 0.05$ versus LPS group, ** $P < 0.01$.

Figure 3: LPS-simulated miR-21 expression depends on AP-1. (A) SYBR Green real-time PCR measurement of pri-miR-21 in A549 cells treated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 hours. Expression was normalized to GAPDH (n=4). (B) SYBR Green real-time PCR detection of dose-dependent

miR-21 expression in A549 cells stimulated with LPS and SR11302. A549 cells are pre-incubated with different concentrations of SR11302 for 30 min including 1, 10, 100, 1000, 10000 nM before LPS (1 μg/ml 12 hours) treatment to measure miR-21 expression. (C) SYBR Green real-time PCR detection of temporal-dependent miR-21 expression in A549 cells stimulated with LPS and SR11302. A549 cells are pre-treated with SR11302 (1uM) for 30min then stimulated with LPS (1 μg/ml) for 4, 6, 12, 24 hours to detect miR-21 expression (n=3). (D) miR-21 expression measured by SYBR Green real-time PCR in A549 cells incubated with LPS (1 μg/ml; 8 hours) after pre-treated with SR11302 (10 nM; AP-1 inhibitor) or LXA_4 (100 nM) for 30 min (n=5). U6 expression is selected as endogenous control for normalization. Relative expression is calculated using the $\Delta\Delta C_t$ method of analysis. * P < 0.05 versus control group, # P < 0.05 versus LPS group.

Figure 4: LXA_4 attenuates LPS-depressed ENaC-γ protein expression through miR-21, but miR-21 does not target ENaC-γ mRNA. (A) SYBR Green real-time PCR and (B) Western blot measurement of ENaC-γ mRNA and protein expression in A549 cells stimulated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 hours (n=4). * P < 0.05 versus control group, # P < 0.05 versus LPS group. (C and D) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic, miR-21 mimic, control inhibitor and miR-21 inhibitor (n=3). (E and F) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic, miR-21 mimic, control inhibitor and miR-21 inhibitor with stimulation of LPS (1 μg/ml; 8 hours) (n=4). * P < 0.05 versus control group, || P < 0.05 versus control mimic, § P < 0.05 versus control inhibitor. (G and H) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic or miR-21 mimic and stimulated with LPS (1 μg/ml) and/or

678 LXA_4 (100 nM) for 8 hours (n=4). * *P* < 0.05. (I and J) Western blot and corresponding densitometry
679 analysis of ENaC-γ protein expression in A549 cells transfected with control inhibitor or miR-21 inhibitor
680 and stimulated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 hours (n=3). * *P* < 0.05. (K)
681 Luciferase/Renilla ratio results for 293T cells cotransfected with miR-21 or control miRNA mimic together
682 with pmiR-RB-SCNN1G CDS (wild type or mutant) for 24 hours.
683
684 **Figure 5**: MiR-21/PTEN pathway responses for LPS and LXA_4 regulation of ENaC-γ. (A) SYBR Green
685 real-time PCR and (B) Western blot measurement of PTEN mRNA and protein expression in A549 cells
686 incubated with LPS (1 μg/ml; 8 hours) after pre-treated with SR11302 (10 nM; AP-1 inhibitor) or LXA_4
687 (100 nM) for 30 min (n=4). (C) Densitometry analysis of PTEN protein expression in B (n=3). * *P* < 0.05
688 versus control group, # *P* < 0.05 versus LPS group. (D and E) Western blot and corresponding
689 densitometry analysis of PTEN protein expression in A549 cells transfected with control mimic, miR-21
690 mimic, control inhibitor and miR-21 inhibitor (n=3). Western blot and corresponding densitometry
691 analysis of PTEN (F and G) and ENaC-γ (H and I) protein expression in A549 cells transfected with control
692 inhibitor or miR-21 inhibitor and stimulated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 hours (n=3).
693 * *P* < 0.05.
694
695 **Figure 6**: LXA_4 augmented LPS decreased ENaC-γ protein expression via miR-21/PTEN/AKT pathway.
696 Western blot and corresponding densitometry analysis of p-AKT (A and B), p-ERK (C and D) and ENaC-γ
697 (E and F) protein expression in A549 cells stimulated with LPS (1 μg/ml; 12 hours) after pre-incubated
698 with SR11302 (10 nM; AP-1 inhibitor), LY294002 (10 μM, PI3K/Akt inhibitor), U0126 (20 μM, ERK
699 inhibitor), or LXA_4 (100 nM) for 30 min (n=3). * *P* < 0.05 versus control group, # *P* < 0.05 versus LPS

group.

Figure 7: Schematic representation of miR-21/PTEN/AKT pathway in LXA_4 regulation of LPS-depressed ENaC-γ protein expression. Through inhibition of PTEN, miR-21 up-regulated phosphorylation of AKT activation therefore reduced the expression of ENaC-γ. However, via inhibiting AP-1 and activating PTEN, LXA_4 reversed LPS-depressed ENaC-γ expression, unveiling a novel therapeutic entry point wherein LXA_4 activated ENaC-γ through down-regulating miR-21 expression.

Figure 1

A

Position 1900-1907 of ATP1B1 3′UTR	5′	TTGTAAGTATTTCTGTGTCGTGGTC
hsa-miR-181a	3′	AACAUUCAACGCUGUCGGUGAGU
Position 1899-1907 of ATP1B1 3′UTR	5′	TTGTAAGTATTTCTGTGTCGTGGTC
hsa-miR-181b	3′	AACAUUCAUUGCUGUCGGUGGGU
Position 169-175 of ATP1B1 3′UTR	5′	UGUAAAUUAAAGUGUAGCAAUAG
hsa-miR-137	3′	GAUGCGCAUAAGAAUUCGUUAUU
Position 169-175 of ATP1B1 3′UTR	5′	TCTTTTACATATTTCGTTTAAT
hsa-miR-137	3′	GGUGUGUAUAAAGUGAGAC
Position 740-746 of ATP1A1 5′UTR	5′	GTCGTGGTCGTACACTACGGAGG
hsa-miR-29a	3′	UAGCACCAUCUGAAAUCGGUUA
Position 740-746 of ATP1A1 5′UTR	5′	GTCGTGGTCGTACACTACGGAGGTC
hsa-miR-29b	3′	UAGCACCAUUUGAAAUCAGUGUU
Position 740-746 of ATP1A1 5′UTR	5′	GTCGTGGTCGTACACTACGGAGGTC
hsa-miR-29c	3′	UAGCACCAUUUGAAAUCGGUUAUG
Position 828-835 of SCNN1G CDS	5′	ATCGAATATGGTGAGGACTTACC
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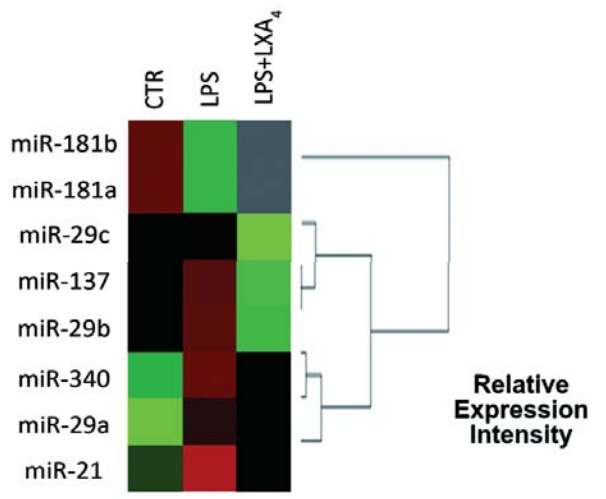
B



C

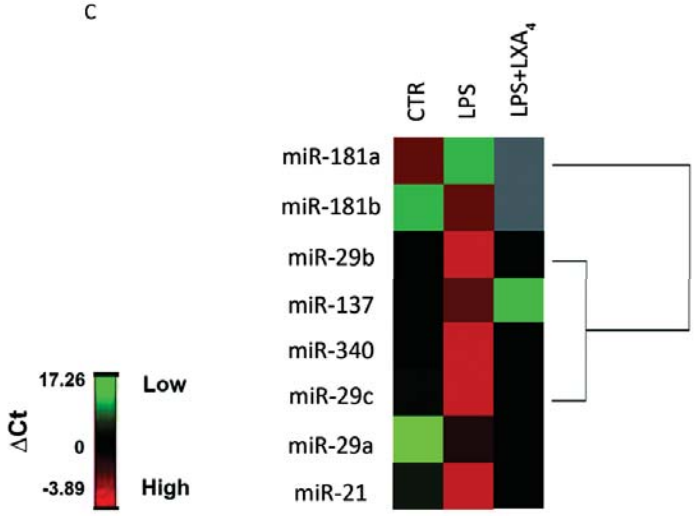
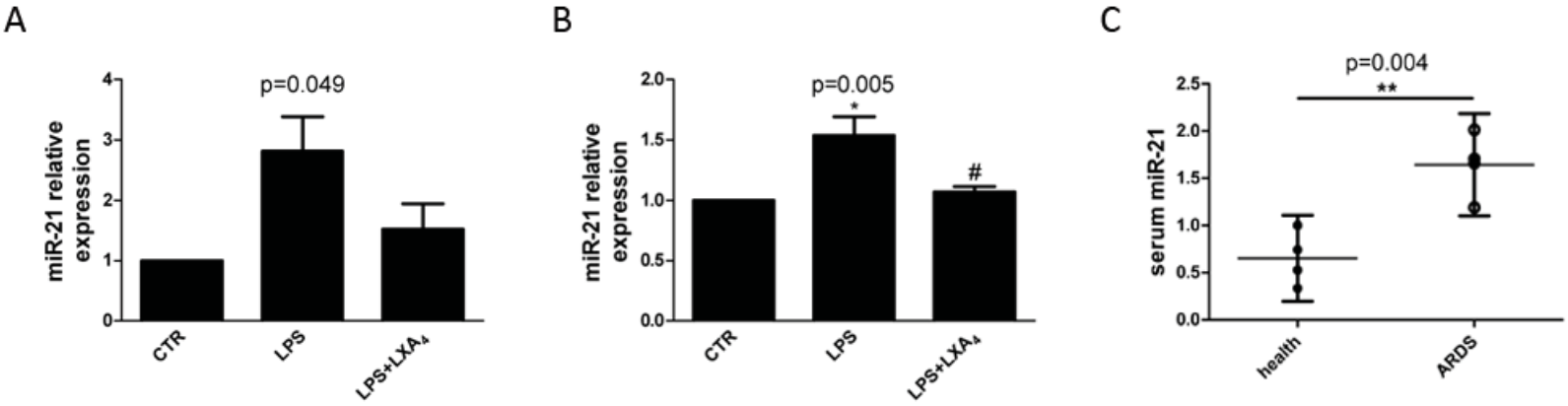


Figure 2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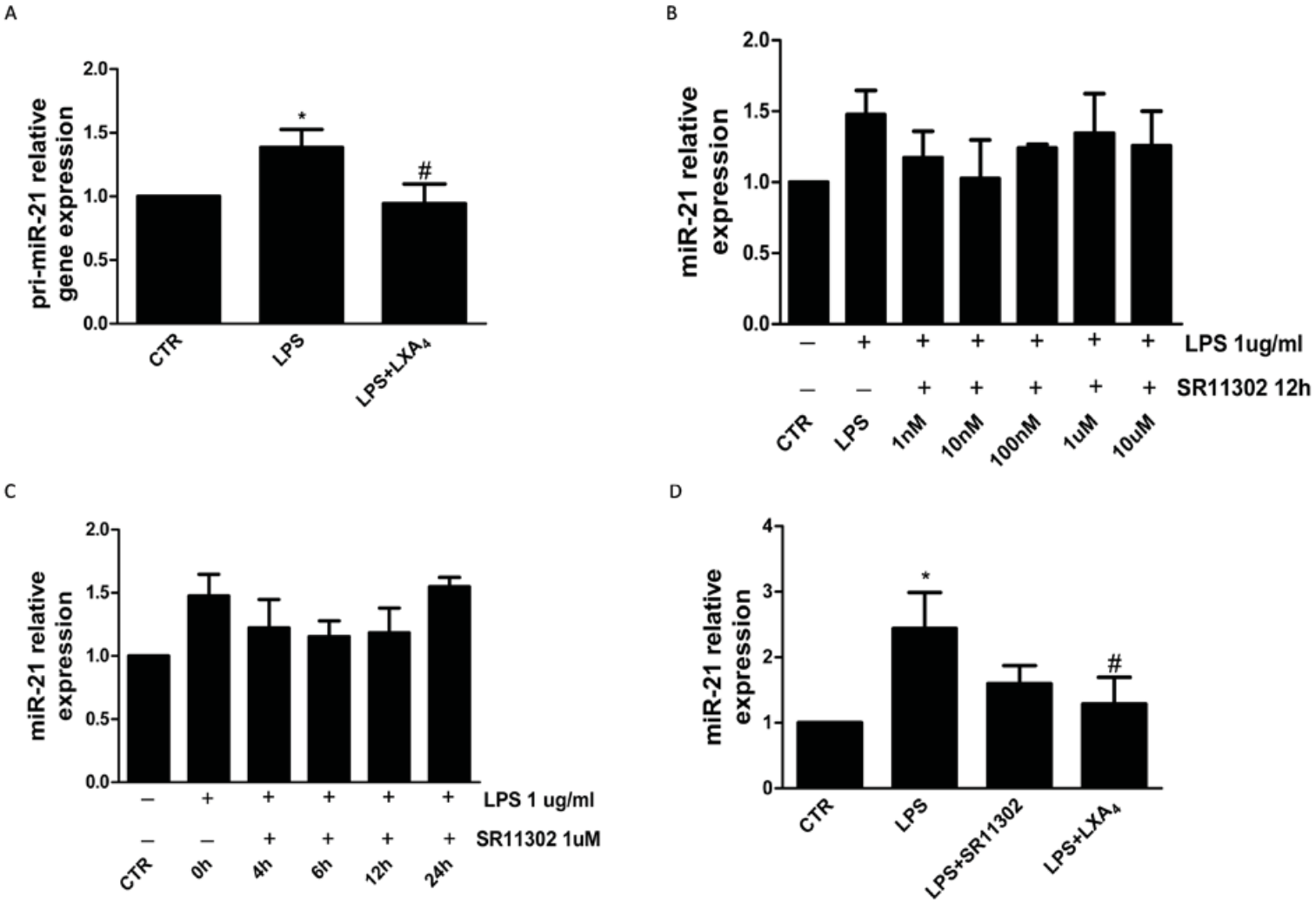
miR-21 relative expression
CTR
LPS
LPS+LXA4
B
p=0.005
*
#
miR-21 relative expression
CTR
LPS
LPS+LXA4
C
p=0.004
**
serum miR-21
health
ARDS

Figure 3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pri-miR-21 relative gene expression
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1.5
1.0
0.5
0.0
*
#
CTR
LPS
LPS+LXA4
B
miR-21 relative expression
2.0
1.5
1.0
0.5
0.0
– + + + + + +
LPS 1ug/ml
– – + + + + +
SR11302 12h
CTR
LPS
1nM
10nM
100nM
1uM
10uM
C
miR-21 relative expression
2.0
1.5
1.0
0.5
0.0
– + + + + +
LPS 1 ug/ml
– – + + + +
SR11302 1uM
CTR
0h
4h
6h
12h
24h
D
miR-21 relative expression
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3
2
1
0
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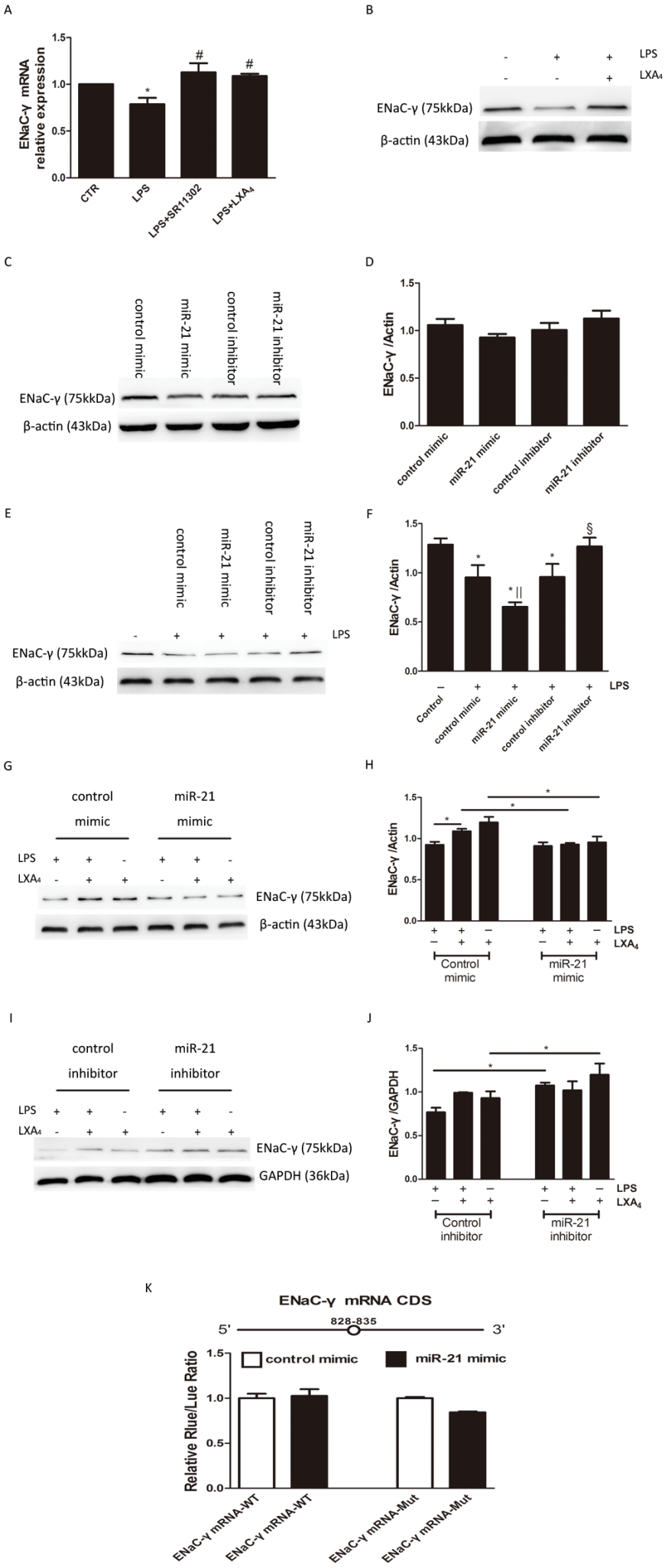
CTR
LPS
LPS+SR11302
LPS+LXA4

Figure 4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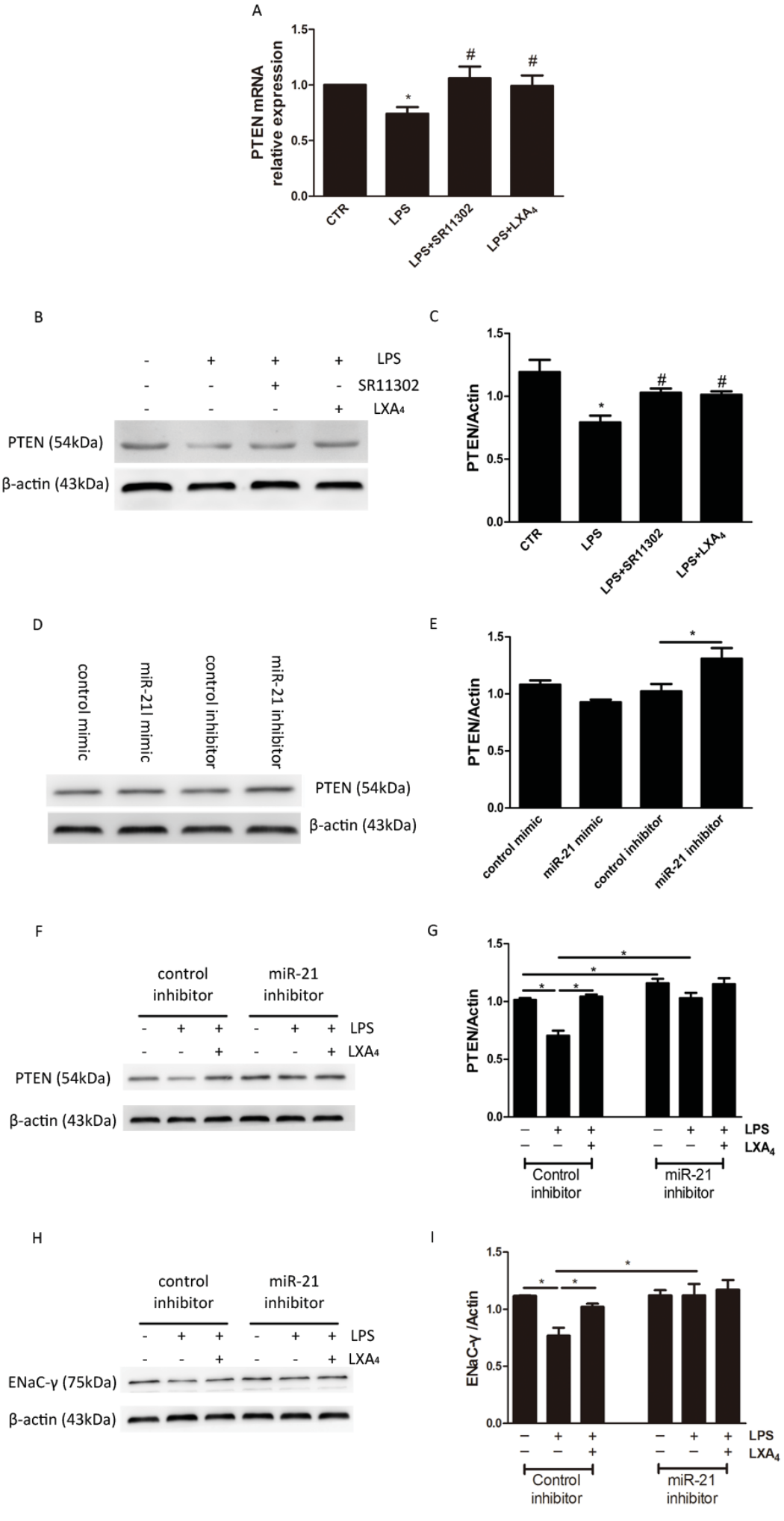
ENaC-γ mRNA relative expression
CTR
LPS
LPS+SR11302
LPS+LXA4
B
LPS
LXA4
ENaC-γ (75kkDa)
β-actin (43kDa)
C
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
ENaC-γ (75kkDa)
β-actin (43kDa)
D
ENaC-γ /Actin
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
E
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
LPS
ENaC-γ (75kkDa)
β-actin (43kDa)
F
ENaC-γ /Actin
Control
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
LPS
G
control mimic
miR-21 mimic
LPS
LXA4
ENaC-γ (75kkDa)
β-actin (43kDa)
H
ENaC-γ /Actin
LPS
LXA4
Control mimic
miR-21 mimic
I
control inhibitor
miR-21 inhibitor
LPS
LXA4
ENaC-γ (75kkDa)
GAPDH (36kDa)
J
ENaC-γ /GAPDH
LPS
LXA4
Control inhibitor
miR-21 inhibitor
K
ENaC-γ mRNA CDS
828-835
5'
3'
control mimic
miR-21 mimic
Relative Rlue/Lue Ratio
ENaC-γ mRNA-WT
ENaC-γ mRNA-WT
ENaC-γ mRNA-Mut
ENaC-γ mRNA-Mut

Figure 5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PTEN mRNA relative expression
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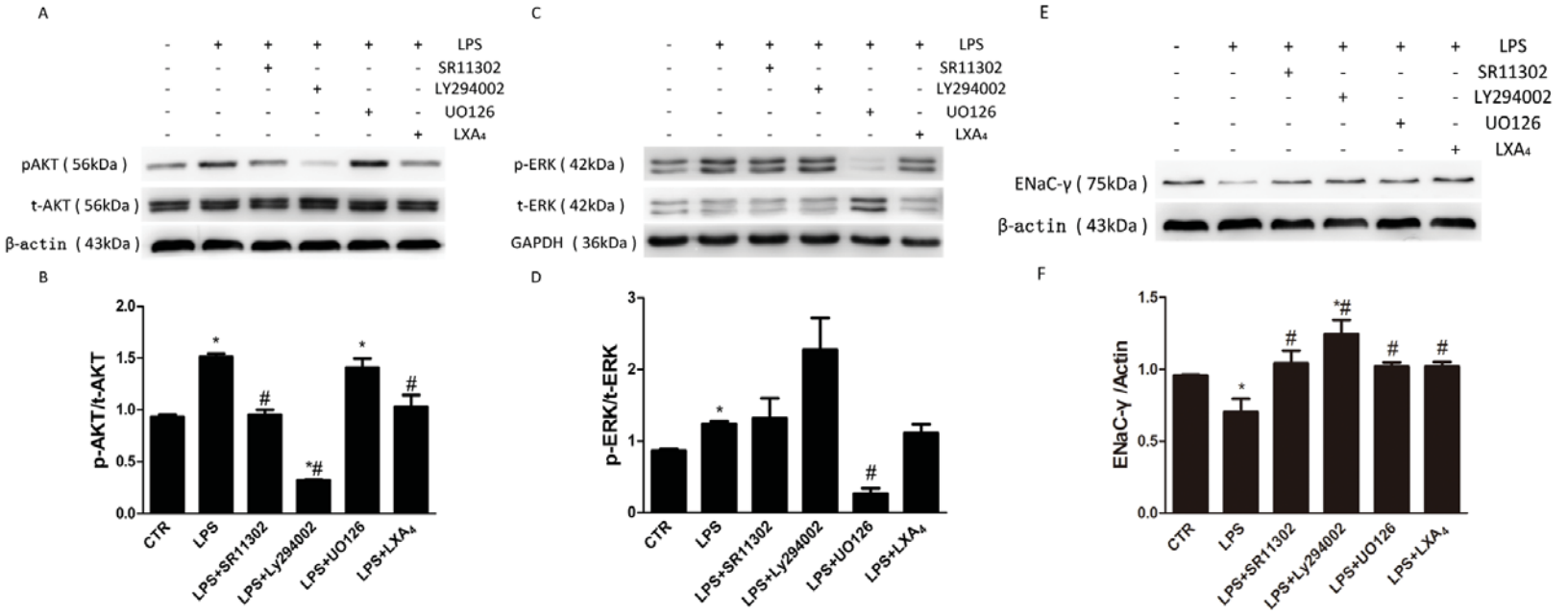
1.0
0.5
0.0
*
#
#
CTR
LPS
LPS+SR11302
LPS+LXA4
B
LPS
SR11302
LXA4
PTEN (54kDa)
β-actin (43kDa)
C
PTEN/Actin
1.5
1.0
0.5
0.0
*
#
#
CTR
LPS
LPS+SR11302
LPS+LXA4
D
control mimic
miR-21l mimic
control inhibitor
miR-21 inhibitor
PTEN (54kDa)
β-actin (43kDa)
E
PTEN/Actin
1.5
1.0
0.5
0.0
*
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
F
control inhibitor
miR-21 inhibitor
LPS
LXA4
PTEN (54kDa)
β-actin (43kDa)
G
PTEN/Actin
1.5
1.0
0.5
0.0
*
*
*
*
LPS
LXA4
Control inhibitor
miR-21 inhibitor
H
control inhibitor
miR-21 inhibitor
LPS
LXA4
ENaC-γ (75kDa)
β-actin (43kDa)
I
ENaC-γ /Actin
1.5
1.0
0.5
0.0
*
*
*
LPS
LXA4
Control inhibitor
miR-21 inhibitor

Figure 6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LPS
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LY294002
UO126
LXA4
pAKT (56kDa)
t-AKT (56kDa)
β-actin (43kDa)
C
p-ERK (42kDa)
t-ERK (42kDa)
GAPDH (36kDa)
E
ENaC-γ (75kDa)
β-actin (43kDa)
B
p-AKT/t-AKT
D
p-ERK/t-ERK
F
ENaC-γ /Actin
CTR
LPS
LPS+SR11302
LPS+Ly294002
LPS+UO126
LPS+LXA4

Figure 7

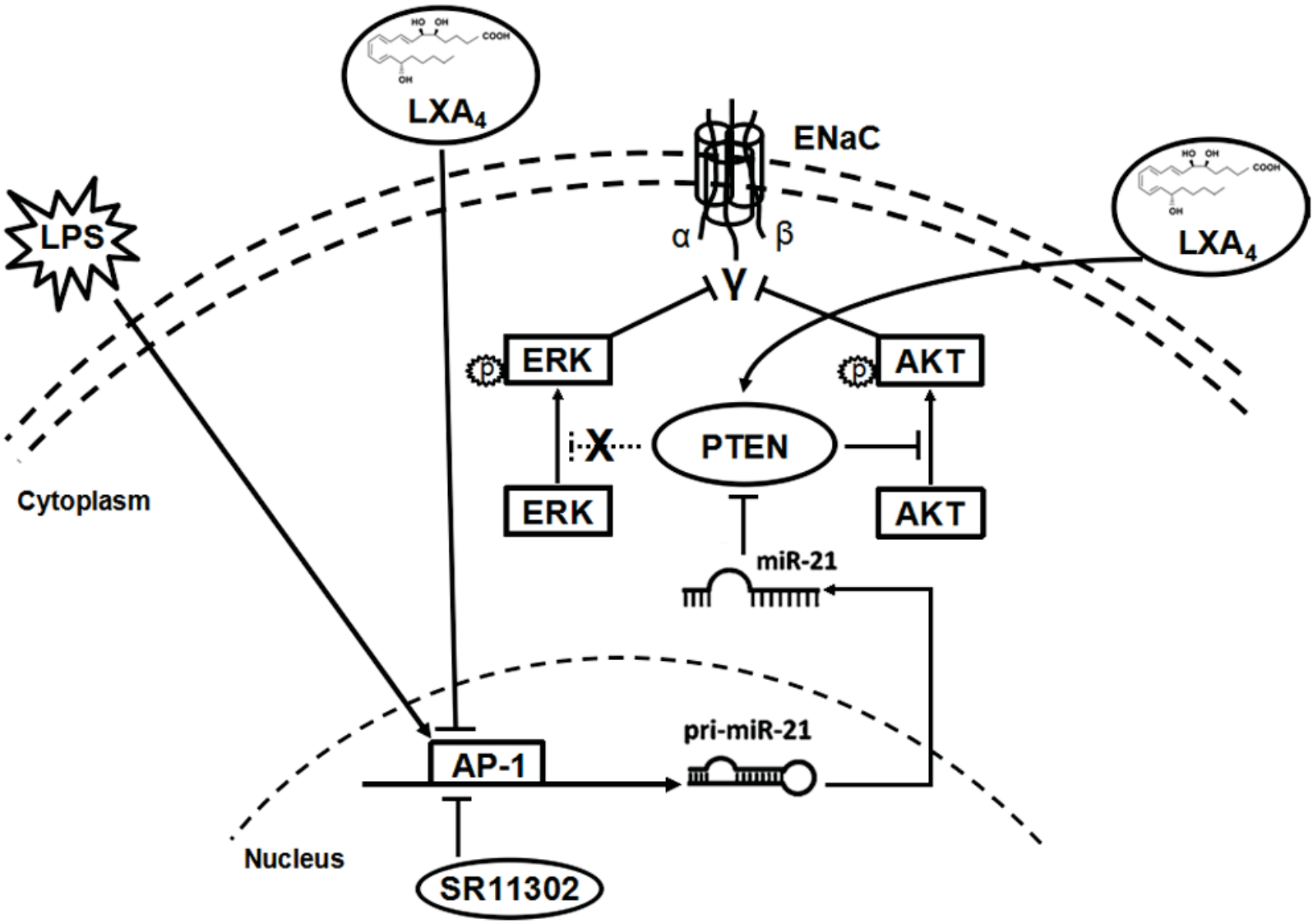
LXA4
ENaC
LPS
α
β
LXA4
ERK
AKT
PTEN
ERK
AKT
miR-21
Cytoplasm
pri-miR-21
AP-1
Nucleus
SR11302

Table S1 Patient Characteristics

Patient	Sex	Age, yr	C-reactive protein (mg/dL)	WBC ($\times 10^9$/ L)	PaO_2/FiO_2 ratio	Clinical Disorder
1	M	59	322	16.5	129	Pneumonia
2	M	86	172	17.3	223	Aspiration of gastric contents
3	F	82	180	27.5	179	Pneumonia and Sepsis
4	M	65	172	13.7	152	Sepsis

M, male; F, female; WBC, white blood cell count.